Summary of Doctoral Thesis

1. Introduction

The fragile X mental retardation protein (FMRP) [*1], a gene product of a causative gene (*FMR1*) for Fragile X syndrome (FXS) [*2], is an RNA binding protein that regulates translation of its target mRNAs. *Fmr1*-knockout (KO) mice, model of FXS, display defects in pre- and post-synaptic structure and plasticity (Bassell & Warren, 2008). Most of the research on FMRP has focused on postsynaptic functions in dendrites, which are regulated by FMRP-dependent local protein synthesis that controls synaptic transmission and plasticity. On the other hand, some researchers recently found less developed presynapse morphology and decreased short-term plasticity in *Fmr1*-KO neurons which are indicative of presynaptic dysfunctions (Klemmer et al., 2011, Deng et al., 2011). However, local translational regulation by FMRP in presynapses of axons still remains unclear. Furthermore, it has not been yet determined which protein(s) are regulated by FMRP-mediated local translation to control presynaptic functions.

Among presynaptic proteins, Munc18-1[*3] (*Stxbp1*gene product), a synaptic vesicle fusion protein, is one of the most interesting candidates as proteins regulated by FMRP-mediated local translation for presynaptic functions (Fig. 1). Munc18-1, a neuronal subtype of Munc18 family, plays an important role in neurotransmitter release from active zone[*4] of presynaptic nerve terminal by binding with SNARE proteins, particularly Syntaxin (Stx). Previously, it is reported that FMRP binds to *Stxbp1* (Munc18-1) mRNA (Darnell et al., 2011), and that mutations in *STXBP1* gene is found in patients in mental retardation, suggesting possible involvement of Munc18-1 in FXS.

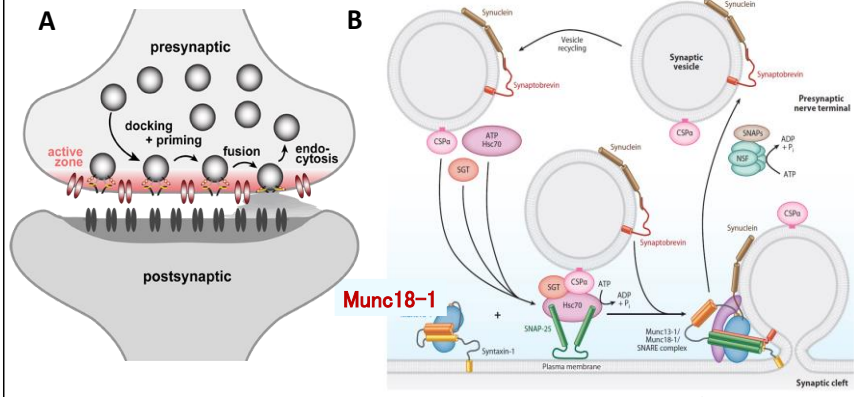

Figure 1. Munc 18-1 is involved in neurotransmitter release in active zones of presynapses. (A). In active zone of presynapses, synaptic vesicles are docked, primed and fused with plasma membrane to release neurotransmitter. (B). Munc18-1 (blue) is involved in synaptic vesicle fusion through SNARE complex formation (bottom right) by interacting with closed conformation of Syntaxin-1 (Habc domain and N-peptide; orange, SNARE motif; yellow) (Rizo & Sudhof, 2012).

In this study, I aimed to clarify whether Munc18-1 is regulated by FMRP-mediated local translation[*5] in presynapses. To do this, I applied presynapse formation assay using beads coated with a presynapse organizer protein, LRRTM2 (Leucine-rich repeat transmembrane neuronal 2) to analyse proteins that accumulate during induced presynapse formation. Using this method, I found that FMRP and Munc18-1 accumulated simultaneously during induced presynapse formation. In *Fmr1*-KO neurons, enhanced level of Munc18-1 accumulation in presynapses was observed, and the enhanced Munc18-1 accumulation was suppressed by a protein synthesis

inhibitor, anisomycin, suggesting suppressional regulation of Munc18-1 by FMRP-mediated local translation in wild type (WT) neuron. Furthermore, in naturally formed synapses in dissociated culture, super resolution fluorescence imaging revealed that Munc18-1 in presynapses of *Fmr1*-KO neurons accumulate more prominently than WT (wild type) at DIV 19 (Days *in vitro*), when FMRP expression reached to peaks in neurites. These findings suggest that transient increase in FMRP during synaptogenesis suppresses earlier accumulation of Munc18-1 to proper level at this stage in WT neurons. Excessive presynapse formation at early stage of synaptic development in *Fmr1*-KO neurons may have a critical role in impaired neural circuit formation and in pathophysiology of FXS and autism.

2. Methods and Materials

Neuron Ball Culture

Neuron ball culture is suitable to observe axonal phonotype, due to formation of dense axonal sheets surrounding cell bodies (Sasaki et al., 2014). Cortical neurons of C57BL/6 or ICR mouse E16 embryo was cultured as hanging drops (10,000 cells/drop) inside the top cover of culture dishes. After 3 days (DIV 3), "neuron balls" were transferred onto poly-L-lysine (PLL)-coated coverslips placed inside 4-well plates, containing Neurobasal media including GlutaMax, B27 supplement (NGB medium). At DIV 11, neuron balls extending neurites up to 1-2 mm are used for experiments (Fig. 2).

Co-culture of dissociated neuron with astroglial feeder layer (Banker method)

We used dissociated neuron culture at low density using Banker method (Kaech and Banker, 2006) to examine naturaly formed individual synapses without so much overlapping. Cortical neurons from E16 mouse cortex were plated at low density (7,000 cells/cm^2) onto PLL-coated, beaded coverslips in 60 mm dishes containing NGB medium for 3 hours to allow cells to adhere firmly on the coverslips. The coverslips with WT and KO cells were transferred onto the feeder layer of glial cells with matched genotypes. This sandwich culture was then maintained in humidified condition in cell incubator up to DIV33.

Preparation of soluble LRRTM2-Fc protein

The cDNA of mouse LRRTM2 extracellular region was amplified by PCR. Then, LRRTM2 cDNA and Human Fc fragment cDNA were inserted to pcDNA3.1 vector. The resulting LRRTM2-Fc and control Fc expression plasmid was transfected to HEK293 cells to establish stable cell lines. Conditioned media from these cell lines were collected, and LRRTM2-Fc and control Fc protein were purified using protein A sepharose beads.

Preparation of LRRTM2-Fc beads

Protein-A magnetic particles (4.0-5.0 μm) were incubated with flag-LRRTM2-Fc in 4°C

chamber for 2 hrs. Blocking with human IgG, rinsing in PBS, and dilution with neurobasal media yielded LRRTM2-Fc beads. LRRTM2-Fc and Fc beads (negative control) are applied on neuron ball culture, and incubated for specified time (30 min to 18 hr) to form presynapses.

Immunofluorescence

Neurons were fixed with 4% paraformaldehyde, washed with PBS, permeabilized with TBS and 0.3% Triton X-100. After 1 hr blocking with IF buffer (TBS, 0.1% Triton X-100, 2% BSA), cells were incubated overnight with 1st antibodies, at 4°C. The 1st antibodies were anti-rabbit-vGlut1 (vesicular glutamate transporter 1; 1:4000), anti-mouse-SYP (Synaptophysin; 1:4000), anti-mouse-Munc18-1 (1:300), anti-mouse-FMRP (clone C3, 1:1000), and anti-rabbit-FMRP (1:200). Coverslips were washed with IF buffer and incubated with fluorophore (Alexa dye)-conjugated 2nd antibodies; anti-mouse-IgG-Alexa488 (1:1000) or -Alexa555 (1:500), and anti-rabbit-IgG-Alexa 488 (1:1000) or -Alexa555 (1:500). Immunofluorescence (IF) images were captured in inverted microscope with CCD camera using 60X oil immersion lens. IF intensity of presynapse was measured using following theory; (Region of Interest on beads (ROI) intensity – Off beads region intensity)/(Axonal intensity along 20 µm from beads – background intensity). To analyze synapses in dissociated neurons, Structured Illumination Microscope (SIM) images were taken using Z-stack 3D SIM mode with 100x oil immersion lens. Statistical significance was analyzed using Student's two-tailed *t* test, Two-way ANOVA with Tukey's multiple comparisons tests; * indicated $P < 0.05$, ** indicated $P < 0.01$ and *** indicated $P < 0.001$, # indicated $P < 0.01$.

2. Results and discussion

Presynapse formation is induced by LRRTM2 beads in axon of neuron ball culture

To analyze the accumulation of presynapstic proteins in axons, I started this study through the formation of presynapses using suitable neuron ball culture and LRRTM2 beads. Here, LRRTM2, a postsynaptic protein that binds with neurexin to induce presynapses in axon, was used as a presynaptic organizer (Fig. 2A). At first to confirm presynapse formation, I evaluated the accumulation of an

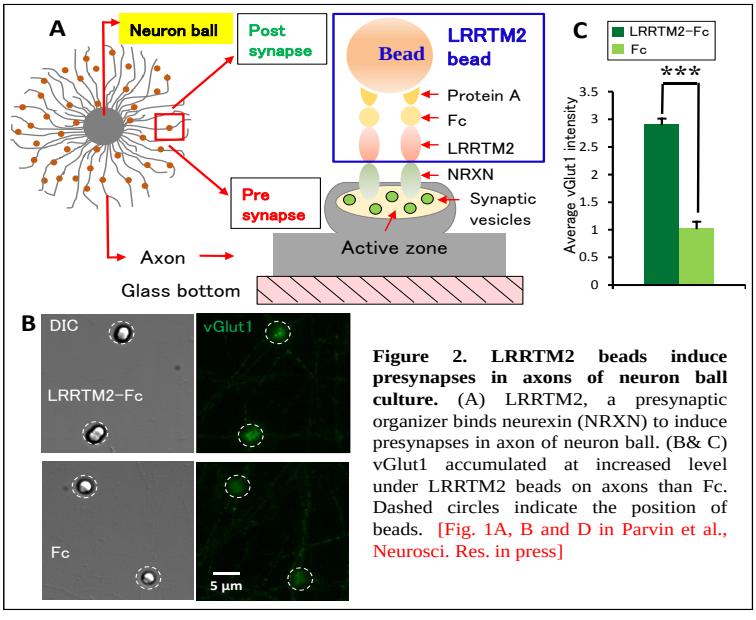

Figure 2. LRRTM2 beads induce presynapses in axons of neuron ball culture. (A) LRRTM2, a presynaptic organizer binds neurexin (NRXN) to induce presynapses in axon of neuron ball. (B& C) vGlut1 accumulated at increased level under LRRTM2 beads on axons than Fc. Dashed circles indicate the position of beads. [Fig. 1A, B and D in Parvin et al., Neurosci. Res. in press]

excitatory synaptic vesicle protein, vGlut1. Figures 2B and C demonstrate that vGlut1 accumulated 3 times more under LRRTM2-Fc beads than control Fc. This data indicates that LRRTM2-Fc beads are very much specific to induce presynapse formation in axon of neuron ball culture. Thus, application of LRRTM2-Fc beads on neuron ball culture is a promising method to investigate the acumulation of presynaptic proteins at induced presynapses in axons.

FMRP accumulated in presynapses in response to LRRTM2 stimulation

For local translation mediated by FMRP in presynapses, FMRP is necessary to localize inside presynapses. However, FMRP localization during presynaptic formation has not been reported yet. Figure 3A illustrates after overnight incubation with LRRTM2-Fc beads, many FMRP-positive puncta accumulated very well with vGlut1 puncta in WT, while no puncta was observed in *Fmr1*-KO presynapses. The observed FMRP puncta in presynapse looks like similar to the presynaptic fragile X granules (FXGs), reported by Christie et al., in intact brain region. These data indicate that FMRP, the RNA binding protein, accumulated in presynapses with high specificity. Times course experiments revealed that FMRP started to accumulate at 2 hr under LRRTM2-Fc beads (Fig. 3B, C), whereas vGlut1 accumulated within 30 min in presynapses (data not shown). This discrepancy of accumulation time between vGlut1 and FMRP suggests that FMRP is not involved in vGlut1 accumulation in presynapses.

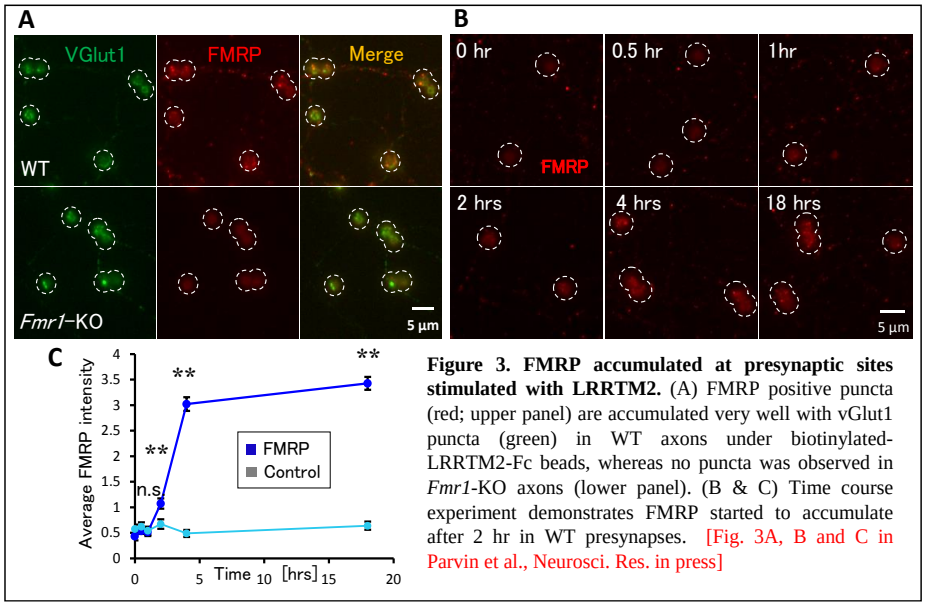

Figure 3. FMRP accumulated at presynaptic sites stimulated with LRRTM2. (A) FMRP positive puncta (red; upper panel) are accumulated very well with vGlut1 puncta (green) in WT axons under biotinylated-LRRTM2-Fc beads, whereas no puncta was observed in *Fmr1*-KO axons (lower panel). (B & C) Time course experiment demonstrates FMRP started to accumulate after 2 hr in WT presynapses. [Fig. 3A, B and C in Parvin et al., Neurosci. Res. in press]

FMRP regulates Munc18-1 accumulation in presynapses

To investigate whether FMRP is involved in Munc18-1 translation in presynapses, I examined Munc18-1 accumulation induced by LRRTM2 beads. LRRTM2 beads incubation at different time points reveals that Munc18-1 accumulated gradually at 2 to 18 hr in WT presynapse (Fig. 4A, B). In presynapses of *Fmr1*-KO neurons, an earlier and profound increase in Munc18-1 accumulation was observed (Fig. 4A, B). These results suggest that FMRP

mediates suppression of Munc18-1 accumulation in WT presynapses. This accumulation time course of Munc18-1 is similar to FMRP accumulation (Figs 3C, 4B). This simultaneous accumulation of FMRP and Munc18-1 leads to the possibility that FMRP directly regulates Munc18-1 translation at presynapses.

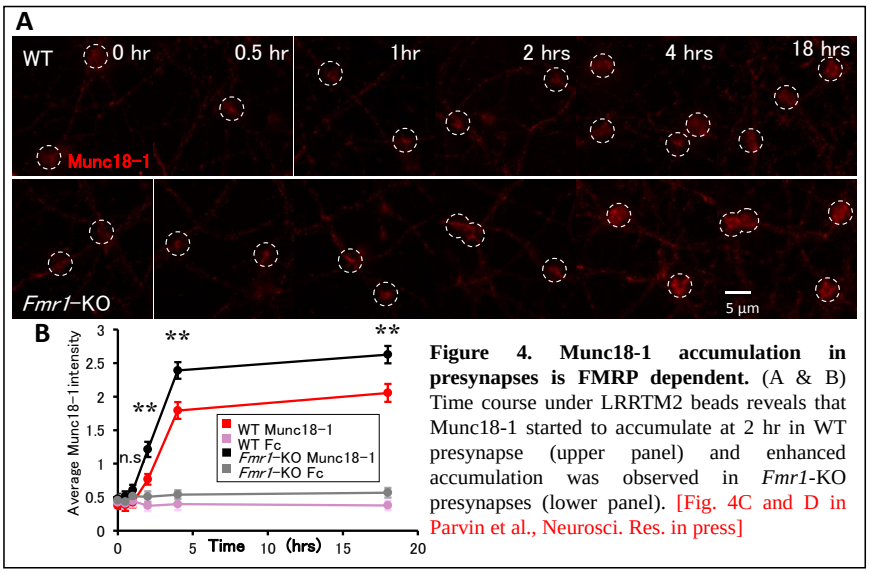

Figure 4. Munc18-1 accumulation in presynapses is FMRP dependent. (A & B) Time course under LRRTM2 beads reveals that Munc18-1 started to accumulate at 2 hr in WT presynapse (upper panel) and enhanced accumulation was observed in *Fmr1*-KO presynapses (lower panel). [Fig. 4C and D in Parvin et al., Neurosci. Res. in press]

Munc18-1 accumulation in presynapses is regulated by FMRP mediated local protein synthesis

To investigate whether Munc18-1 accumulation is due to FMRP-mediated local translation, I examined an effect of protein synthesis inhibitor (anisomycin) on Munc18-1 accumulation in presence and absence of cell bodies in WT and *Fmr1*-KO presynapses (Fig. 5A). Munc18-1 accumulated in WT presynapses even in absence of cell bodies. This accumulation was significantly reduced by anisomycin (Fig. 5A, B), indicating that Munc18-1 accumulation in WT presynapses is controlled by local translation in axons. Moreover, enhanced accumulation of Munc18-1 in *Fmr1*-KO presynapses (with or without cell bodies) were significantly suppressed by anisomycin (Fig. 5A, B). These findings suggest that FMRP regulates local translation of Munc18-1 negatively in presynapses.

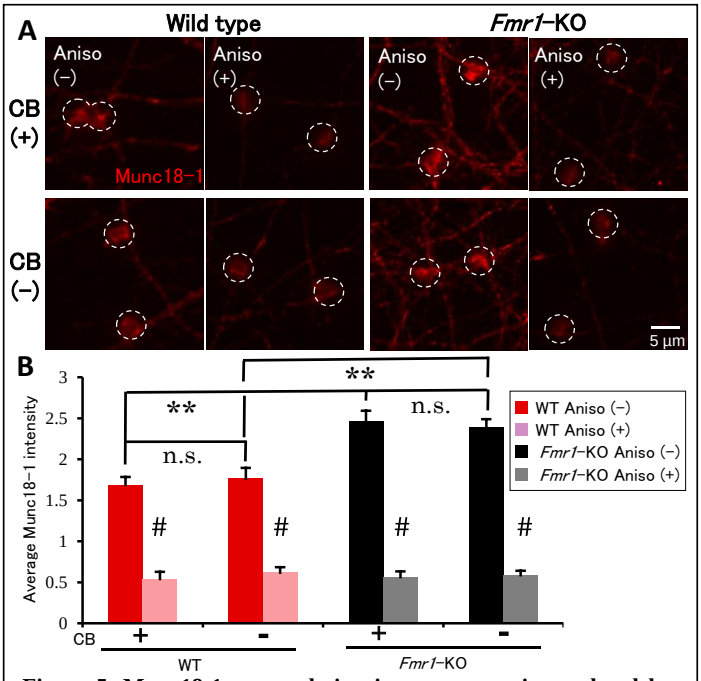

Figure 5. Munc18-1 accumulation in presynapse is regulated by FMRP mediated local translation. (A & B) Munc18-1 accumulation (red) in WT presynapses (left panel) in presence and absence of cell bodies is suppressed by anisomycin. Enhanced accumulation in *Fmr1*-KO presynapses (right panel) even without cell bodies, is suppressed by anisomycin. Aniso; Anisomycin, CB; Cell body.
[Fig. 5B and C in Parvin et al., Neurosci. Res. in press]

FMRP regulates Munc18-1 accumulation in naturally formed synapses

To investigate whether Munc18-1 accumulation in naturally formed synapses is under regulation of FMRP or not, I examined expression level of these proteins in neurites and synapses of low-density dissociated culture prepared by the Banker method. The accumulation of Munc18-1 increased significantly in KO neurites than WT around DIV19, however, reached to same level of WT at DIV33 (Fig 6A). On the other hand, FMRP expression in WT neurites increased until DIV 19 and gradually declined after this stage, suggesting that the transient increase in FMRP around DIV 19 suppresses earlier accumulation of Munc18-1 at this stage in WT neurons (Fig 6A). Super-resolution fluorescence image analysis of DIV 19 neurites reveals Munc18-1, not vGlut1, accumulation intensity in puncta increased significantly in KO neuron (Fig. 6B-D). Moreover, Munc18-1 accumulation in synapses (defined by merged areas with vGlut1 puncta) was upregulated more in KO neurites (Fig. 6B, E). From these results, I hypothesize that FMRP suppresses Munc18-1 expression to proper level during synaptogenesis. My results are consistent with the translatome analysis of retinal axon by Shigeoka et al. They showed that FMRP expression level is high at DIV 17.5 in retinal ganglion cell axons, and that FMRP downregulates translation of its target proteins during this synaptogenesis period. Taken together, FMRP may regulate specific synaptic proteins to proper level for synapse formation.

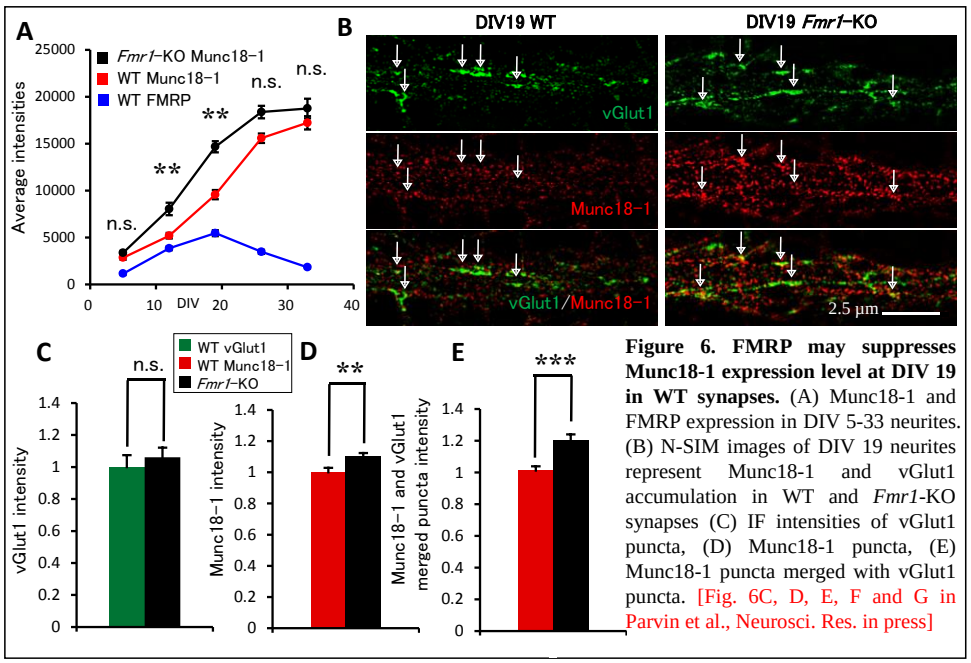

Figure 6. FMRP may suppresses Munc18-1 expression level at DIV 19 in WT synapses. (A) Munc18-1 and FMRP expression in DIV 5-33 neurites. (B) N-SIM images of DIV 19 neurites represent Munc18-1 and vGlut1 accumulation in WT and *Fmr1*-KO synapses (C) IF intensities of vGlut1 puncta, (D) Munc18-1 puncta, (E) Munc18-1 puncta merged with vGlut1 puncta. [Fig. 6C, D, E, F and G in Parvin et al., Neurosci. Res. in press]

Conclusion and possible model of FXS pathophysiology related to Munc18-1

In this study, I present three lines of evidence to demonstrate that FMRP-regulated local translation in axons is involved in accumulation of the active zone protein Munc18-1 in presynapses during LRRTM2-induced excitatory presynapse formation: 1) FMRP and Munc18-1 accumulates in presynapses at the same time i.e. at 2-4 hr after stimulation by

LRRTM2-Fc beads in WT axons (Figs 3 & 4), 2). Munc18-1 accumulation in presynapses increased more in *Fmr1*-KO axons than WT axons during presynapse formation by LRRTM2 stimulation (Fig. 4), and 3). The Munc18-1 accumulation in presynapses occurs even in axons physically separated from cell bodies of neuron ball culture (Fig. 5). To the best of my knowledge, it is the first report to demonstrate involvement of RNA-binding proteins in local translation of presynaptic proteins in axons. In addition, Munc18-1 accumulation is significantly increased at DIV 19 in naturally-formed synapses of *Fmr1*-KO neurons in dissociated culture. This accumulation is correlated to transient increase of FMRP accumulation at DIV 19 of WT neurites. These results suggest that FMRP regulates negatively Munc18-1 expression for proper level during synapse formation.

Previously, Verhage et al., have shown that neurotransmitter release was blocked in *Munc18-1/Stxbp1*-KO neocortices in E18 mice. Enhanced synaptic vesicle release rate is also reported by Deng et al. in *Fmr1*-KO presynapse around DIV 14-18. Thus, on the basis of crucial link between Munc18-1 expression levels to neurotransmitter release, I propose following model for FXS pathophysiology (Fig. 7). FMRP and its translational target Munc18-1 accumulated at presynapses during synaptogenesis. Binding of FMRP to Munc18-1 mRNA may control its translation to proper level by FMRP-mediated suppression in WT neurons, so that leads to controlled glutamate release. In *Fmr1*-KO neuron, lacking of FMRP regulation results excessive local translation of Munc18-1 in axons and increased glutamate release (Fig. 7). This excessive Munc18-1 accumulation and increased synaptic activity at early stage of *Fmr1*-KO neurons could explain pathophysiology of FXS and autism. Here, it may be possible to consider Munc18-1 as a key component of FX pathologic pathway. Suppression of expression and/or activity of presynaptic proteins such as Munc18-1 may help to develop new therapeutic approaches to improve the symptoms of FX patients, and these approaches may provide significant advantages in treatment for FX and other related developmental disorders. Much more additional research will be required to develop these therapeutic approaches for clinical use.

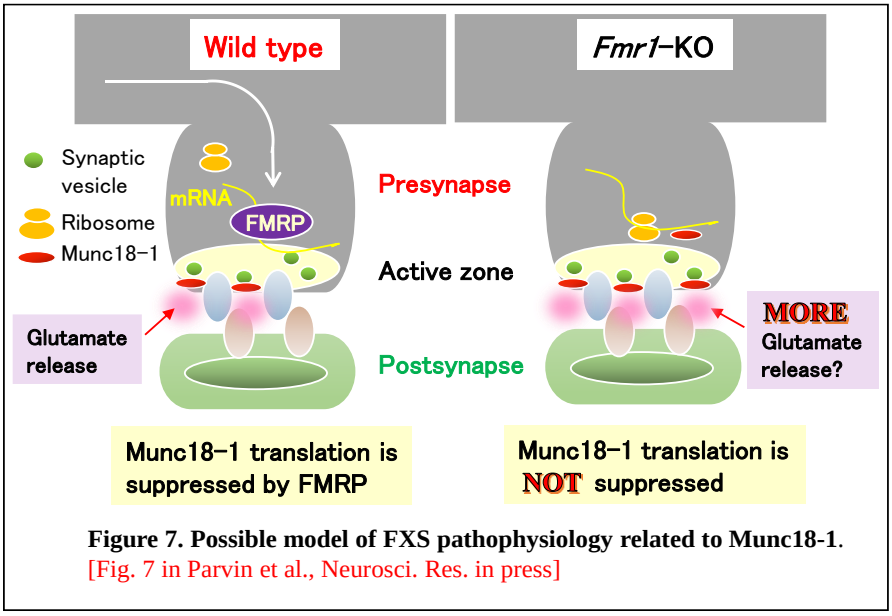

Figure 7. Possible model of FXS pathophysiology related to Munc18-1.
[Fig. 7 in Parvin et al., Neurosci. Res. in press]

3. Summary

1) FMRP and its translational target Munc18-1 accumulated at the same time in LRRTM2 beads-stimulated presynaptic sites in axons, and Munc18-1 accumulated more in *Fmr1*-KO presynapses.
2) The Munc18-1 accumulation in WT and KO presynapses even in the absence of cell body fractions of neuron balls decreased significantly by anisomycin, suggesting that FMRP negatively regulates local translation of Munc18-1 in axons.
3) Munc18-1 accumulated more in *Fmr1*-KO neurites and synapses than those of WT around DIV 19 in dissociated culture when FMRP expression in WT neurites was also reached to maximum, suggesting negative regulation effects of FMRP on Munc18-1 translation in WT synapses.

7. Publication

Shumaia Parvin, Renoma Takeda, Yu Sugiura, Makiko Neyazaki, Terukazu Nogi, and Yukio Sasaki. "Fragile X mental retardation protein regulates accumulation of the active zone protein Munc18-1 in presynapses via local translation in axons during synaptogenesis." *Neuroscience Research* (2018). *In press*.

8. References

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9. Glossary

[*1] **FMRP:** FMRP (fragile X mental retardation protein) having amino acid length of 632 is a highly conserved RNA binding protein composed of KH domain, RGG box, and Agenet motif. It binds with approximately 5% of all brain mRNAs and non-coding RNAs (miRNA, BC1). FMRP regulates mRNA stability, mRNA transport along neurites, and translation inside synapses to control synaptic functions.

[*2] **Fragile X syndrome:** Fragile X syndrome (FXS) is a neurodevelopmental disorder, characterized by intellectual disability and autistic features. FXS is caused by CGG repeat expansion in the 5'-untranslated region of *Fmr1* gene on X chromosome, resulting in low level or no expression of FMRP. Some neurological phenotypes include mental retardation, autism, attention deficit hyperactivity disorder (ADHD), anxiety, instability, repetitive behaviour, epilepsy etc.

[*3] **Munc18-1:** Munc18-1 (nSec1, Munc18-a, rbSec1), a member of Sec1/Munc18-related proteins, is involved in synaptic vesicle docking, priming, and fusion, as a key component of neurotransmitter release. These functions are mediated by binding to neuronal SNARE proteins, especially Syntaxin-1.

[*4] **Active zone:** Active zone is a site in presynaptic nerve terminal that mediates synaptic vesicle fusion and neurotransmitter release, essential for synaptic function. It is composed of highly conserved proteins including RIM, Munc13, Munc18, RIM-BP, SNARE proteins etc. Upon arrival of action potential, these proteins help in Ca^{2+} triggering, priming synaptic vesicles, and keeping active zone exactly in opposite position to postsynapse.

[*5] **Local translation:** Local translation is a phenomenon in which specific mRNAs are transported to specialized neuronal compartments and translated in response to localized signal. Neuron can store numerous mRNAs and translate them only when required to produce specific cellular response. Local translation of mRNAs into proteins plays a vital role in proper neuronal function and synaptic plasticity.